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Gene Flow Between Great Lakes Region Populations of the Canadian Tiger Swallowtail Butterfly, *Papilio Canadensis*, Near the Hybrid Zone With *P. Glaucus* (Lepidoptera: Papilionidae)

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GENE FLOW BETWEEN GREAT LAKES REGION POPULATIONS OF THE CANADIAN TIGER SWALLOWTAIL BUTTERFLY, *PAPILIO CANADENSIS*, NEAR THE HYBRID ZONE WITH *P. GLAUCUS* (LEPIDOPTERA: PAPILIONIDAE)

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ABSTRACT

Papilio canadensis were sampled from three locations on either side of Lake Michigan to study gene flow near and through a butterfly hybrid zone. Allele frequencies at four polymorphic enzyme loci, as indicated by allozyme electrophoresis, were similar in all samples. Values for F_{ST} were close to zero, indicating that gene flow is high among these populations, even when separated by Lake Michigan. We developed a mitochondrial DNA marker with diagnostic differences between *P. canadensis* and its parapatric sister species *Papilio glaucus*, based on PCR-RFLP. *P. glaucus* haplotypes of this mtDNA marker and *P. glaucus* alleles of a diagnostic allozyme locus (PGD) were found in *P. canadensis* populations sampled in Michigan's Lower Peninsula but not in the Upper Peninsula or Northern Minnesota. The presence of *P. glaucus* alleles in *P. canadensis* populations could be due to introgression through hybridization, or could be remnants of a *P. glaucus* population that was inundated by an influx of *P. canadensis* alleles.

Gene flow, the passage of alleles from one population to another, is one of the most important factors determining the ecology and microevolution of a species (Slatkin 1987). When gene flow is high, advantageous alleles spread quickly to new populations. Limited gene flow, however, promotes local adaptation when a species' range is spread across heterogeneous environments and increases the chance that drift will lead to allele frequency differences between populations. This is the basis for methods, such as Wright's *F*-statistics, that use differences of presumably neutral molecular or morphological markers between populations to estimate genetic structure and infer levels of gene flow (Roderick 1996).

Introgression is the presence of genetic characteristics of one species within some individuals of another species. Patterns of introgression provide information about evolutionary history and the nature of the barriers between populations. Studying gene flow within each of two hybridizing species is also important to understanding the barriers between them, as stronger isolating mechanisms are needed to maintain differences when levels of dispersal and gene flow are high (Barton & Hewitt 1985).

The Canadian Tiger Swallowtail butterfly *Papilio canadensis* (Rothschild and Jordan) (Lepidoptera: Papilionidae) was originally described as a subspecies of the parapatric Eastern Tiger Swallowtail *Papilio glaucus* (Linn.) (Lepidoptera: Papilionidae). Recently, on the basis of differences in a number of genetic traits including adult and larval morphological characters, diapause induction, abilities to use different larval food plants, and three enzyme loci

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detectable by allozyme electrophoresis, they have been classified as distinct species (Hagen et al. 1991). The opportunity for interbreeding exists between *P. canadensis* and *P. glaucus* because their ranges meet in a hybrid zone (Figure 1) and their flight times overlap (Scriber 1996). Lab-produced hybrids are viable and fertile (Hagen and Scriber 1995), and the barriers to gene flow or the selective forces that maintain the distinctness of the two species are poorly understood. Allozyme markers have indicated that gene flow among *P. glaucus* populations is high, (Bossart and Scriber 1995), but gene flow within *P. canadensis* has not been studied. One approach to studying gene flow is to estimate levels between populations separated by natural barriers such as mountain ranges or large bodies of water. For example, if gene flow was high between *P. canadensis* populations separated by one of the Great Lakes, then it is likely that gene flow would be high between populations separated by large geographic distances. The isolating mechanisms between *P. canadensis* and *P. glaucus* would also have to be very strong to maintain the observed distinctness of the two species.

To estimate levels of gene flow within *P. canadensis*, we sampled *P. canadensis* populations on either side of Lake Michigan. Allozyme electrophoresis was used to determine allele and genotype frequencies at four polymorphic enzyme loci. A diagnostic PCR-RFLP mitochondrial genetic marker was developed and used to compare *P. glaucus* mitochondrial introgression with nuclear introgression at the PGD locus in these same populations.

MATERIALS AND METHODS

Gene flow among *P. canadensis* populations. Butterflies were collected by net from six locations in the range of *P. canadensis* in the Great Lakes region: one from northeast Minnesota (Cook Co.: 35 males), two from the Upper Peninsula of Michigan (Gogebic Co.: 36 males, 1 female; Dickinson Co.: 48 males, 20 females), and three from the Lower Peninsula of Michigan (Charlevoix Co.: 50 males, 17 females; Mason Co.: 50 males, 15 females; Isabella Co.: 50 males, 14 females) (Fig. 1). All specimens were collected in 1998 between 14 May and 23 June (peak flight time for *P. canadensis* in Michigan), and stored at -80°C.

Specimens were prepared by grinding the distal half of the abdomen for males or the proximal half of the abdomen for females (to avoid including spermatophore proteins) in 100μL buffer (0.1M tris, 1.07mM EDTA, 0.15mM NAD, 0.13mM NADP, 35.75mM 2-mercaptoethanol, pH 7.0) and centrifuging for 10 minutes at 14,000 rpm. Allozyme electrophoresis was carried out on thin layer cellulose acetate plates (Titan III, Helena Laboratories, Beaumont, TX) following Hagen & Scriber (1991). The four enzymes surveyed, (GPI, PGM, HBDH, and PGD), were selected for ease of scoring and polymorphism within *P. canadensis* (Hagen & Scriber 1991). The electrophoresis conditions for each are shown in Table 1.

Enzyme stains followed Richardson et al. (1986). Gels were scored as in Hagen and Scriber (1991). The most common allozyme for each enzyme was given a score of '100', the origin (where samples had originally been applied) was given a score of '0', and all other allozymes were scored by their location relative to these two points (the relative migration distance score). Every sample plate was run with at least two previously scored samples to act as internal standards. The relative migration distance scores were then used as names for different alleles at the enzyme gene locus. Because preserved specimens previously scored for GPI, PGM, and HBDH were not available, the names given to these allozymes are not consistent with those given in Hagen and Scriber (1991). Since comparisons to previous studies were possible for HK, LDH, and PGD, the allozyme names used here are consistent with those used in Hagen and Scriber (1991), Hagen et al. (1991), and Scriber et al. (1998) (although in Scriber et al. (1998) PGD^{-125} was called PGD^{90} and PGD^{-80} was called PGD^{110}).

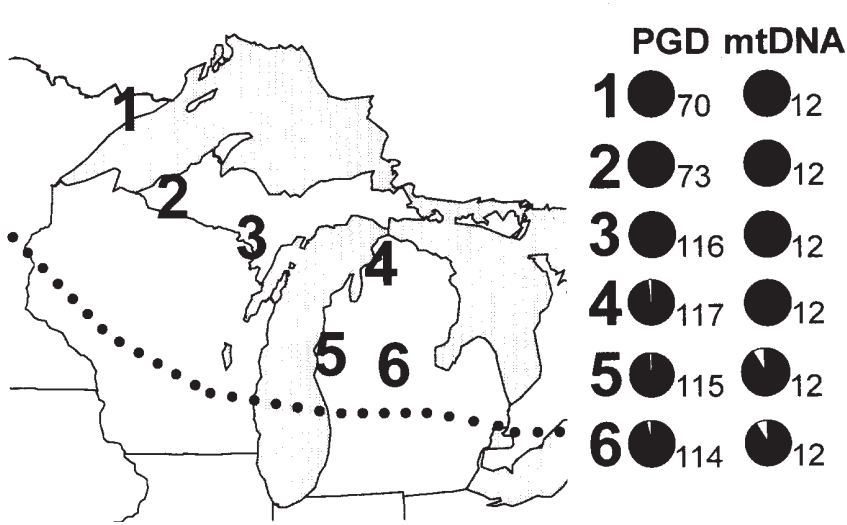

Figure 1. Sample sites for *P. canadensis* and introgression of *P. glaucus* alleles. 1. Cook Co., Minnesota (35 males). 2: Gogebic Co., Michigan (36 males, 1 female). 3: Dickinson Co., Michigan (48 males, 20 females). 4: Charlevoix Co., Michigan (50 males, 18 females). 5: Mason Co., Michigan (50 males, 15 females). 6: Isabella Co., Michigan (50 males, 14 females). Dotted line shows the northernmost extent of the hybrid zone between *P. canadensis* and *P. glaucus* as indicated by the collection of dark-morph females (Scriber 1996). Dark portion of pie graphs show the frequency of *P. canadensis* alleles or haplotypes, open portion of graphs show the frequency of *P. glaucus* alleles or haplotypes. Numbers to the right of graphs are numbers of alleles or haplotypes sampled from each population.

Table 1. Enzymes resolved and running conditions used.

Enzyme	Name (E.C. Number)	Buffer[1]	Origin	Voltage	Time
GPI	Glucose phosphate isomerase (5.3.1.9)	I	cathode	275V	45 min.
HBDH	Hydroxybutyrate dehydrogenase (1.1.1.30)	D	anode or cathode[2]	300V	90 min.
HK	Hexokinase (2.7.1.1)	D	cathode	300V	45 min.
LDH	Lactate dehydrogenase (1.1.1.27)	D	cathode	300V	60 min.
PGM	Phosphoglucomutase (2.7.5.1)	I	cathode	275V	45 min.
PGD	6-phosphogluconate dehydrogenase (1.1.1.44)	D	anode or cathode[2]	300V	90 min.

[1] Buffers (as in Richardson et al. 1986): I=25mM tris, 192mM glycine, pH 8.5; D=15mM tris, 5mM EDTA, 10mM $MgCl_2$, 5.5mM boric acid, pH 7.8.
[2] Under these conditions, HBDH and PGD migrated towards the center of the plate regardless of origin.

Genepop v3.1 (Raymond and Roussett 1995) was used to test for linkage disequilibrium between loci and to perform exact tests for allele frequency differences between populations. Fstat v2.8 (Goudet 1995) was used to calculate Wright's *F*-statistics and standard errors.

Development of a species diagnostic PCR-RFLP mitochondrial marker. From each specimen, two legs were plucked for DNA extraction. Methods used followed Sperling & Hickey (1995). PCR primer sites and restriction sites were developed from *P. canadensis* and *P. glaucus* sequences of the mitochondrial cytochrome oxidase I gene (COI) (Genbank accession numbers AF044014 and AF044013) (Caterino & Sperling 1999). The PCR primers used are shown in Figure 2, and were expected to produce a DNA fragment 284 base pairs long in individuals of both species. PCR was carried out with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) according to their recommended conditions in a total reaction volume of 100µl with 10 pmoles of each primer and 2µl extracted DNA. Amplification conditions were 36 cycles of 94°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes followed by a final extension of 72°C for 5 minutes. Amplification of PCR products was verified on 2% agarose gels along with a 123 bp DNA ladder.

PCR products were then incubated separately with the restriction enzymes *Taq*I (for 120 minutes at 65ºC) and *Ssp*I (for 60 minutes at 37ºC). It was expected that *Taq*I RE would cut the PCR product from *P. glaucus* individuals (into fragments of 217 and 67 base pairs) but not from *P. canadensis* individuals (Fig. 2). It was also expected that *Ssp*I RE would cut the PCR product from *P. canadensis*

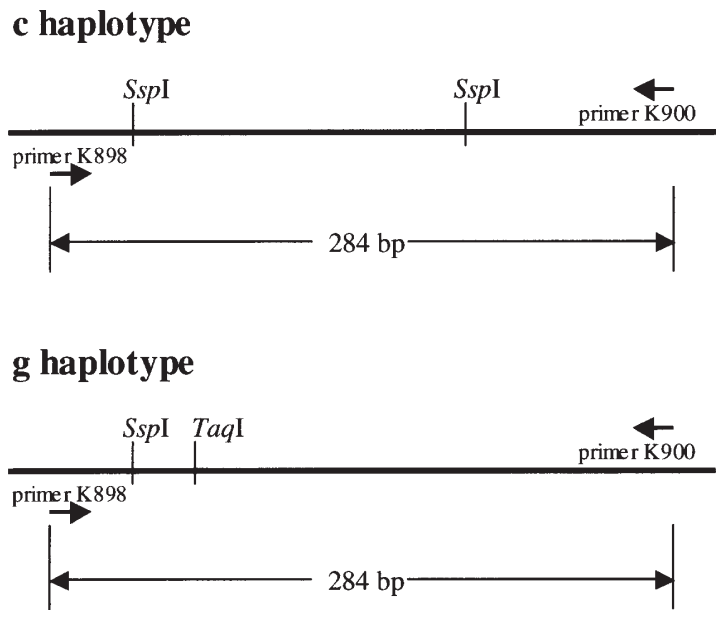

Figure 2. Restriction map for PCR fragment of mitochondrial COI gene amplified in this study. Primer K898 had sequence 5' ATA ATT GGA GGA TTT GGA AAT TG 3' and the location of the 3' end is homologous to base pair 1709 in the published sequence of the COI gene of *Drosophila yakuba* (Clary & Wolstenholme 1985). Primer K900 had sequence 5' ATT GTA GTA ATA AAA TTA ATT GCT CC 3' and the 3' end is homologous to bp 1945 of *D. yakuba*. See text for sizes of restriction fragments after digestion with *Ssp*I or *Taq*I.

Table 2. Diagnostic mtDNA haplotypes for *P. canadensis* and *P. glaucus* as visualized by PCR-RFLP.

Species	Collection Location	Collection Date	*Ssp*I digested[3]	*Taq*I digested[3]	haplotype
P. canadensis female	Fairbanks, Alaska	June 1995	3	1	c
P. canadensis female	Fairbanks, Alaska	June 1995	3	1	c
P. canadensis male	Fairbanks, Alaska	June 1995	3	1	c
P. canadensis female	Thunder Bay, Ontario	June 1995	3	1	c
P. canadensis male	Thunder Bay, Ontario	June 1995	3	1	c
P. canadensis female	Pancake Bay, Ontario	June 1995	3	1	c
P. canadensis male	Bayfield Co., Wisconsin	June 1995	3	1	c
P. canadensis male	Forest Co., Wisconsin	June 1995	3	1	c
P. canadensis male	Mackinac Co., Michigan	June 1996	3	1	c
P. canadensis female	Charlevoix Co., Michigan	June 1995	3	1	c
P. canadensis male	Manistee Co., Michigan	June 1995	3	1	c
P. canadensis female	Isabella Co., Michigan	June 1996	3	1	c
P. glaucus male	St. Joseph Co., Michigan	July 1995	2	2	g
P. glaucus male	St. Joseph Co., Michigan	July 1995	3	1	c
P. glaucus female D1	Lawrence Co., Ohio	September 1995	2	2	g
P. glaucus female Y2	Lawrence Co., Ohio	September 1995	3	1	c
P. glaucus male	Wise Co., Virginia	August 1994	2	2	g
P. glaucus male	Wise Co., Virginia	August 1994	2	2	g
P. glaucus male	Clarke Co., Georgia	August 1995	2	2	g
P. glaucus female D1	Clarke Co., Georgia	August 1995	2	2	g
P. glaucus female Y2	Clarke Co., Georgia	August 1995	2	2	g
P. glaucus male	Highlands Co., Florida	September 1995	2	2	g
P. glaucus female D1	Highlands Co., Florida	September 1995	2	2	g
P. glaucus female Y2	Highlands Co., Florida	September 1995	2	2	g

[1] D=dark morph *P. glaucus* female
[2] Y=yellow morph *P. glaucus* female
[3] Numbers refer to the number of restriction digestion products. See text for details.

individuals at two sites (giving fragments 148, 98, and 38 bps long) but would only cut *P. glaucus* fragments at one site (into 246 bp and 38 bp fragments). The length polymorphisms of digested DNA, when electrophoresed on 2% agarose gels, were then used to differentiate *P. canadensis* haplotypes from *P. glaucus*. Twelve *P. canadensis* and twelve *P. glaucus* from fourteen geographic locations, all collected prior to 1997 and stored at -80°C (Table 2) were used to verify that the primers would amplify a DNA fragment of the correct size and that the restriction sites were appropriate for use as a species diagnostic.

***P. glaucus* alleles in *P. canadensis* populations.** PGD, in addition to being polymorphic within *P. canadensis* populations, has diagnostic differences between *P. canadensis* and *P. glaucus*. The survey of allozyme frequencies in the 1998 samples of six locations Michigan and Minnesota (Fig. 1) provided an estimate of the frequencies of P. glaucus PGD allozymes within each of these P. canadensis populations. This meant that the survey of allozyme frequencies in the 1998 samples from six locations in Michigan and Minnesota (Fig. 1) revealed an estimate of the frequencies of *P. glaucus* PGD allozymes within each of these *P. canadensis* populations. Twelve males were also randomly chosen from each of these six samples and for each individual the mtDNA haplotype was determined by PCR-RFLP as described previously. Additionally, the mtDNA haplotypes of all individuals carrying *P. glaucus* PGD alleles were determined, and all individuals carrying *P. glaucus* PGD or mtDNA alleles were genotyped for two other diagnostic allozyme loci (HK and LDH, Table 1).

RESULTS

Gene flow among *P. canadensis* populations. There were no significant P-values indicating linkage disequilibrium between any two loci (Table 3), suggesting that all four loci can be taken to be independent sources of information. Allozyme frequencies were similar in all six P. canadensis populations for all four loci, but there were some significant differences between some populations at all four loci (Table 4). Significant overall allozyme frequency differences were found for GPI (P=0.004), and marginally significant overall differences were found for PGD (P=0.06). Nevertheless, there was no general pattern of differentiation of populations separated by Lake Michigan. Neighboring populations were as likely to be different as distant populations. Two populations with allozyme frequencies significantly different at one locus were not generally different at other loci.

Wright's F-statistics for these six populations are shown in Table 5. All F_{ST}-values were less than 0.01. F_{ST} for PGM was calculated to be less than zero, and for the other three enzymes, F_{ST} was within its standard error's range of zero. This indicates that there is little significant reduction in heterozygosity due to population subdivision.

Development of a species diagnostic PCR-RFLP mitochondrial marker. All 24 butterflies selected to verify the PCR-RFLP diagnostic marker had PCR products slightly shorter than 300 bp long, which is the size expected from the COI gene sequence. No individual produced two PCR fragments.

The PCR products of all twelve *P. canadensis* individuals were digested into three fragments by *Ssp*I and none were cut by *Taq*I (Table 2). The PCR products of ten of the twelve *P. glaucus* individuals were cut only once by *Ssp*I and were also cut by *Taq*I. The other two *P. glaucus* PCR products were cut as the *P. canadensis* products were. We define the absence of a *Taq*I restriction site in this region of the COI gene and the presence of two *Ssp*I sites as the c haplotype and the presence of a *Taq*I site and a single *Ssp*I site as the g haplotype (Figure 2).

***P. glaucus* alleles in *P. canadensis* populations.** For the 1998 population samples, relative frequencies of P. canadensis *and* P. glaucus PGD allozymes and mtDNA haplotypes are shown in Figure 1. PGD allozymes typical of P. glaucus were found in the three Lower Peninsula P. canadensis population

Table 3. Chi-square values from tests of linkage of enzyme loci. The null hypothesis was H_0: genotypes at one locus are distributed independently from genotypes at the other locus.

Locus pair	$\chi 2$	df	*P*-value
GPI & PGM	9.845	12	0.630
GPI & HBDH	10.307	12	0.589
PGM & HBDH	4.386	12	0.975
GPI & PGD	14.229	12	0.286
PGM & PGD	9.555	12	0.655
HBDH & PGD	17.025	12	0.149

samples, all at frequencies lower than 0.1 (PGD^{-100} at 0.009 in Charlevoix Co., 0.009 in Mason Co., and 0.035 in Isabella Co.; PGD^{-50} at 0.009 in Charlevoix Co., Table 4). Mitochondrial DNA typical of P. glaucus was found only in the Mason and Isabella samples (one out of twelve individuals, frequency equal to 0.083 for both). No *P. glaucus* mtDNA was found in either Michigan Upper Peninsula population sample or the northern Minnesota population sample.

Of the two *P. canadensis* individuals carrying *P. glaucus* mtDNA, one carried no *P. glaucus* allozymes from any of the diagnostic allozyme loci (PGD, LDH, HK), and the other carried a typical *P. glaucus* allozyme allele only for HK and was heterozygous at that locus (Table 6). Six of seven individuals with typical *P. glaucus* PGD allozymes had no other *P. glaucus* allozymes at either of the other enzyme loci or in their mtDNA. The seventh carried a *P. glaucus* HK allozyme (again heterozygous) but not at the other loci. Thus within individuals, the occurrence of *P. glaucus* allozymes from one locus was not usually coincidental with *P. glaucus* alleles at other loci.

DISCUSSION

Our allozyme data, producing F_{ST}-values very close to zero (Table 5), indicate that there is little or no genetic differentiation between the *P. canadensis* populations studied, even populations separated by Lake Michigan and distances in the hundreds of kilometers. Some statistically significant allozyme frequency differences were found (Table 4), but they were always small frequency changes. Because of the lack of consistent differences between populations separated by Lake Michigan, and because differences at one locus are not also found at other loci, we believe that these small differences are consistent with the F_{ST}-supported finding of overall genetic homogeneity. These results are similar to those found for *P. glaucus* (Bossart and Scriber 1995), and suggest that these *Papilio* species are mobile and long-lived enough to maintain significant levels of gene flow. The results could also reflect a problem with the resolution of allozymes (Bossart and Prowell 1998).

When looking only at one allozyme locus with diagnostic differences between *P. canadensis* and *P. glaucus* (PGD, a sex-linked gene) and mtDNA (also with diagnostic species differences), a clear, if minor, structure can be seen in the Great Lakes *P. canadensis* populations sampled (Fig. 1). Individuals were found with typical *P. glaucus* PGD allozymes, and others were found with the typical *P. glaucus* mtDNA haplotype, but only in the Lower Peninsula populations (Fig. 1). There are two possible explanations for the presence of *P. glaucus* alleles in *P. canadensis* populations in the observed geographic pattern. It could be due to the introgression of *P. glaucus* alleles into *P. canadensis* populations through hybridization. This would explain why *P. glaucus* alleles are found in the two populations closest to the northern extent of the range of *P. glaucus* (Isabella and Mason Counties) but not in three populations farther away. However, Charlevoix County,

Table 4. Allozyme frequencies for GPI, HBDH, PGD, and PGM from six *P. canadensis* populations. The rows labeled sample size indicate the number of allozymes resolved for each population. In the rows labeled statistics, populations not sharing a letter are significantly different at $P = 0.05$, and the column labeled overall shows the *P*-value for the null hypothesis H_0: the allozyme distribution is identical across populations.

Locus	Allozyme	Cook	Gogebic	Dickinson	Charlevoix	Mason	Isabella	Overall
GPI	-100	0.0	0.0	0.015	0.007	0.008	0.0	
	0	0.186	0.095	0.074	0.127	0.138	0.055	
	100	0.743	0.797	0.809	0.836	0.815	0.898	
	150	0.029	0.014	0.0	0.0	0.0	0.0	
	200	0.043	0.095	0.103	0.030	0.038	0.031	
	300	0.0	0.0	0.0	0.0	0.0	0.016	
	sample size	70	74	136	134	130	128	
	statistics	A	ABC	B	AC	ABC	C	$P = 0.004$
PGM	60	0.029	0.014	0.059	0.015	0.031	0.031	
	67	0.086	0.108	0.103	0.060	0.100	0.125	
	85	0.371	0.365	0.382	0.388	0.408	0.414	
	100	0.386	0.351	0.346	0.321	0.338	0.352	
	115	0.100	0.162	0.110	0.164	0.100	0.063	
	122	0.029	0.0	0.0	0.015	0.008	0.0	
	130	0.0	0.0	0.0	0.030	0.008	0.016	
	140	0.0	0.0	0.0	0.007	0.008	0.0	
	sample size	70	74	136	134	130	128	
	statistics	AB	AB	A	B	AB	A	$P = 0.561$
HBDH	30	0.0	0.014	0.0	0.0	0.0	0.0	
	45	0.029	0.014	0.037	0.022	0.046	0.047	
	60	0.0	0.0	0.015	0.007	0.008	0.008	
	100	0.886	0.797	0.882	0.888	0.885	0.898	
	160	0.043	0.135	0.037	0.060	0.062	0.016	
	170	0.043	0.014	0.029	0.022	0.0	0.023	
	180	0.0	0.0	0.0	0.0	0.0	0.008	
	sample size	70	74	136	134	130	128	
	statistics	AB	A	AB	AB	AB	B	$P = 0.185$
PGD	-150	0.029	0.0	0.069	0.026	0.035	0.0	
	-137	0.0	0.0	0.0	0.0	0.009	0.0	
	-125	0.886	0.863	0.828	0.906	0.878	0.904	
	-100	0.0	0.0	0.0	0.009	0.009	0.035	
	-90	0.0	0.0	0.009	0.0	0.0	0.0	
	-80	0.086	0.137	0.095	0.051	0.070	0.061	
	-50	0.0	0.0	0.0	0.009	0.0	0.0	
	sample size	70	73	116	117	115	114	
	statistics	AB	AB	A	AB	AB	B	$P = 0.060$

Table 5. Wright's *F*-statistics for six *P. canadensis* populations through the Great Lakes region. Standard errors were obtained by jackknifing over populations, and are indicated in parentheses.

Locus	F_{IS} (s.e.)	F_{ST} (s.e.)	F_{IT} (s.e.)
GPI	0.002 (0.015)	0.009 (0.009)	0.011 (0.016)
PGM	0.051 (0.044)	-0.004 (0.002)	0.046 (0.043)
HBDH	0.108 (0.076)	0.002 (0.008)	0.110 (0.078)
PGD	-0.016 (0.039)	0.005 (0.007)	-0.011 (0.039)

Table 6. *P. canadensis* individuals from 1998 samples carrying *P. glaucus* alleles or haplotypes. *P. glaucus* alleles are in parentheses.

Individual	mtDNA	PGD	LDH	HK
Charlevoix male 44	c	-125/(-50)	80/80	(100)/110
Charlevoix female 10	c	(-100)	80	110/110
Mason male 2	c	-125/(-100)	80/80	110/110
Mason male 21	(g)	-125/-125	80/80	110/110
Isabella male 5	(g)	-125/-125	80/80	(100)/110
Isabella male 13	c	-125/(-100)	80/80	110/110
Isabella male 43	c	-125/(-100)	80/80	110/110
Isabella female 10	c	(-100)	80	110/110
Isabella female 13	c	(-100)	40	110/110

where *P. glaucus* alleles were also found, is no closer to this border than the other three sites where no introgression was found. The other possibility is that *P. glaucus* was formerly found throughout all of the Lower Peninsula, and that an influx of *P. canadensis* inundated the populations with *P. canadensis* alleles, and the *P. glaucus* alleles that are found there now are remnants of the old populations. This would explain why *P. glaucus* alleles were found only in the Lower Peninsula. Currently we cannot choose between these two possibilities but the explanation of introgression seems simpler than that of genetic remnants of old populations. It is assumed that *P. glaucus* alleles in *P. canadensis* populations represent introgression through hybridization.

A primary (or F_1) hybrid *Papilio*, produced by a mating of a mother fixed for the diagnostic characters of one species with a father fixed for those of another species, will have a very specific pattern of these genetic characters. Autosomal genes will be heterozygous, a mix of characters from both species. In males, Z-linked genes will be heterozygous, and females will carry the Z-linked genes of the father's species (in butterflies, females are the heterozygous sex and the sex chromosomes are named Z and W, with females being ZW and males ZZ). All offspring will carry the mtDNA characters of the mother's species. This pattern was not found in any of the individuals we found carrying *P. glaucus* alleles (Table 6). Typically, we found individuals to have *P. glaucus* alleles at only one locus. This indicates that when present, the genetic mixing is at least two generations old and has since been maintained in *P. canadensis* populations.

The presence of *P. glaucus* mtDNA suggests that some introgression was produced by *P. canadensis* males mating with *P. glaucus* females, and that female hybrid offspring survived and in turn reproduced, despite a significant Haldane Effect for this cross (Hagen and Scriber 1995). Mitochondrial introgression between species has been previously found in insects (Aubert and Solignac 1990,

Powell 1983), but usually the Haldane effect affects males, making females a more likely bridge for introgression. In butterflies, females are affected by the Haldane effect, but they can evidently still be a bridge for hybridization (Sperling 1993, Sperling 1994).

It is interesting that one out of twelve individuals from both Mason and Isabella Counties had the *P. glaucus* mtDNA haplotype which may indicate that mitochondrial introgression is present at higher frequencies than nuclear introgression. Because the sample sizes characterized for mtDNA for each population are so small (n=12), further sampling would be needed to confirm mtDNA introgression bias. Further sampling would also be needed to determine how far north *P. glaucus* mtDNA can be found, such as whether it is present in Charlevoix County. It also remains to be seen how much *P. canadensis* introgression is present in *P. glaucus* populations. We found c haplotypes in *P. glaucus* individuals from 1995 in St. Joseph Co., Michigan and Lawrence Co., Ohio (one out of two samples from each location, Table 2). However, all twelve *P. glaucus* collected in Lawrence Co., Ohio in 1998 (the same year as our larger *P. canadensis* sampling) had the g haplotype (A. Stump, unpublished data). St. Joseph Co., Michigan is very near the hybrid zone with *P. canadensis*, so it may have high levels of *P. canadensis* introgression. These two samples do not indicate that the c and g haplotypes are not representative markers for these two species. They are simply indicators that genetic mixing occurs.

It has been suggested (Scriber 1988) that the speciation of *P. glaucus* and *P. canadensis* occurred in allopatry during the Wisconsin glaciation from 75,000 to 10,000 years ago, with *P. glaucus* developing south of the glaciation and *P. canadensis* developing in the Beringial refuge in what is now Alaska. The retreat of the glaciers allowed the ranges of the differentiated populations to meet, where they now form a hybrid zone. Previous results (Hagen 1990, Hagen et al. 1991, Luebke et al. 1988, Scriber et al 1998) and those here suggest that when the two species met, barriers to interbreeding were strong but not complete. Thus, it is possible to find individuals with a blending of the traits of the two species, especially near the hybrid zone.

Hybrid zones are defined as being maintained by a balance between migration and selection (either against hybrids or against species having crossed a species boundary) (Barton 1979). Our allozyme data and those of Bossart and Scriber (1995) indicate that these butterflies are capable of significant dispersal, so the counteracting selection must be very strong to maintain the observed differentiation. What could produce this selection? It has been found that *P. glaucus* males prefer conspecific females to *P. canadensis* (Deering and Scriber 2002), but the same study found that *P. canadensis* males prefer *P. glaucus* females to conspecifics. This might mean that if a *P. glaucus* immigrant into *P. canadensis* territory is a male, it will find few attractive mates and thus be unlikely to reproduce (reducing introgression), but if it is a female, the local males will be more attracted to it than to other females, increasing introgression. This pattern of male mating preference predicts that there would be differences in introgression on either side of the hybrid zone: higher mtDNA introgression on the *P. canadensis* side than on the *P. glaucus* side, which would be an interesting direction for future study. Interestingly, female selection of male mates could be more important than male choice but has not been investigated in this context.

Even after pairing has occurred, it is still possible for prezygotic isolation to occur, a phenomenon called conspecific sperm precedence (Howard 1999). This can be due either to cryptic female choice (Eberhard and Cordero 1995) or to sperm competition (Parker 1970). However, there is no postpairing, prezygotic isolation between *P. canadensis* and *P. glaucus* (Stump 2000).

Postzygotic isolation between species can be divided into endogenous and exogenous factors (Jiggins and Mallet 2000), with the former being physiological

in nature and the latter ecological and behavioral. Hybrid eggs hatch at a rate equal to eggs of parental species (Stump 2000), hybrid larvae develop in the lab as quickly as parentals and do not suffer higher mortality even at a range of growing conditions (Donovan 2001), and hybrid adults have been found (at least in some cases) to be fertile, as indicated by the ability to produce backcross offspring (Hagen and Scriber 1995, Stump 2000) and F_2 offspring (A. Stump, unpublished data), (although there have been no studies measuring adult hybrid fertility as compared to parental fertility). The one place that an endogenous barrier has been found is in pupae. There females with a *P. glaucus* mother and a *P. canadensis* father are more likely to die in the pupal stage, the Haldane effect, (Hagen and Scriber 1995), but it would only affect one quarter of the potential types of primary hybrids. It is not even a complete barrier as some of these hybrid adults emerge and can reproduce.

Exogenous selection is more difficult to study but could be very important to keeping these species isolated. In the north, second generation individuals carrying *P. glaucus* diapause genes may emerge in the late summer where there is not enough time for another full generation to reach the diapausing pupal stage, and be selected against by frost or declining leaf quality. Differences in host-plant use ability might also exclude *P. glaucus* genes from *P. canadensis* areas.

There have been many questions in recent years of how global warming could affect the ecology of species. Climate change has been implicated in the range shift of a checkerspot butterfly (Parmesan 1996) and in changes in a genetically controlled ecological character (photoperiod response) in the pitcherplant mosquito (Bradshaw and Holzapfel 2001). Global warming might shift the range of *P. glaucus* (or specific traits) northward, especially if diapause and generation timing is a critical factor in range determination (Scriber 2002a, 2002b). This might also result in increased introgression and a widening of the hybrid zone, rather than a simple northward shift of the trait clines. The data of this paper could provide a baseline against which to compare future samples.

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